African Journal of Biotechnology Vol. 10(13), pp. 2493-2502, 28 March, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.1870
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Applied orthogonal design for filtrating conditions of ultrasonic-assisted extraction from plant-chicory

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Accepted 14 March, 2011

The objective of the current study is to achieve global optimization of ultrasound-assisted extraction from chicory roots using a mixed orthogonal array design. Eight conditional factors were examined in the mixed orthogonal array (L_{16} ($4^3 \times 2^6$)). The results showed that the importance of the eight factors, in decreasing order, was ethanol content, impregnation repetitions, ultrasonic input power, sonication temperature, sonication repetitions, solvent-to-solid ratio, impregnation time and sonication time. The optimum extraction conditions included a frequency of 40 kHz, an impregnation time of 24 h with two rounds of impregnation, a sonication period of 30 min and an ultrasonic input power of 400 W with two rounds of sonication. Importantly, these conditions were independent of alcohol content, solvent-to-solid ratio and sonication temperature. At frequency of 40 kHz, the alcohol content, solvent-to-solid ratio and sonication temperature were optimized in the range of 50 to 75% (v/v), 32:1 and 50°C, respectively.

Key words: Chicory roots, ultrasound-assisted extraction, mixed orthogonal design, select factors, global optimization.

INTRODUCTION

Chicory (*Cichorium intybus* L.) is a perennial plant of the Asteraceae family and is native to the Mediterranean region, Central Asia and Northern Africa. It was first cultivated as a medicinal plant and a vegetable crop in ancient Rome and Greece (Plmuier, 1972). Today, it is popularly cultivated in Europe and North America and has many commercial uses. For example, the shoots and leaves are grown for consumption in salads and vegetable dishes (Hocking and Withey, 1987), as forage crops (Labreveux et al., 2006) and as raw material for fructose and spice production (Ricca et al., 2009), while the roots are dried and roasted for use as a coffee substitute (Bais and Ravishankar, 2001) and feed additive (He et al., 2002). There are a variety of chicory varieties that are utilized for different purposes for example, Grasslands Puna was the original specie (Rumball, 1986); Puna II was intended for use as a pure sward on non-milking farms and as a component of mixed swards on all grazing farms (Rumball et al., 2003); Accalai is an annual plant that require each year, while Marrubiu was able to regrow and to persist (Sulas, 2004). Sheep discriminates against forage feast, while deer like eating INIA LE Lacerta (Labreveux, 2002) and choice fit to culture in winter (Rumball et al., 2003).

Previous studies on chicory have focused on cultivation (Foster et al., 2006), management of growth, yield or quality (Arya et al., 1990) and breeding (Red'ko et al., 2008). However, in places where chicory is cultivated commercially (Cavin et al., 2005), studies have focused extensively on physiological and biochemical activities of extracts of the chicory leaf and root, which has potential for medical use, including polysaccharides and anthocyanins in the leaves (Vilkhu et al., 2008), compounds

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Abbreviations: γ-TMT, Gamma-tocopherol methyltransferase; **UAE,** ultrasound-assisted extraction.

with antitumor activity (Hazra et al., 2002), components that reduce intestinal absorption of glucose (Kim and Shin, 1996), potential antifungal agents (Mares et al., 2005), components that promote active fermentation and lactobacilli proliferation in the large intestine and components that promote calcium digestion and deposition in bones (Demigne et al., 2008). Additionally, natural chicory has excellent bifidogenic effects, as its inulin and hydrolysis products are efficiently fermented by intestinal bacteria (Roberfroid et al., 1998).

In addition to commercial uses, chicory has been combined with molecular biotechnologies. For example, the gamma-tocopherol methyltransferase (γ-TMT) gene, which encodes an enzyme that converts γ--tocopherol and γ--tocotrienol into their alpha (α) isoforms, was introduced into the chicory genome (Hwang et al., 2009). Additionally, an efficient method for plant regeneration and agrobacterium-mediated transformation with the interferon- α 2b gene has also been developed for chicory (cv. *Pala rossa*) with a regeneration efficiency close to 100% (Matvieieva et al., 2009). Moreover, a mixture of roasted chicory roots and wheat germ (1:1 w/w) subjected to extrusion processing has been developed to make a high quality and stable coffee substitute (Fadel et al., 2008). In addition, β-carbolines in chicory and coffee were shown to be beneficial for Parkinson's disease patients and also partially reduced suicide risk with moderate consumption (Alves et al., 2009).

Ultrasound-assisted extraction (UAE) has been widely utilized to isolate bioactive substances from different parts of plants (Dong et al., 2010; Heo and Kim, 2010; Riera et al., 2010) in addition to its many other applications (Molero et al., 2010; Molero et al., 2009; State et al., 2010; Sun et al., 2010; Zhang and Li, 2010). This process can accelerate the dissolution process (Zhang et al., 2009) for high-efficiency extractions (Chen et al., 2009) (e.g., extracting salvianolic acid B from *Salvia miltiorrhiza* root (Dong et al., 2010)). The efficiency of this process is influenced by many extraction conditions, including power, frequency, temperature and time of extraction. However, the reported methods have a wide range of optimized conditions. For example, studies have used the frequency and power combinations of 25 kHz and 150 W (Cuoco et al., 2009; Rodrigues and Pinto, 2007), 35 kHz and 320 W (Ozcan et al., 2009) and 20 kHz and 450 W (Roldan Gutierrez et al., 2008); temperatures from 25 to 90°C (Table 7) and extraction times from 5 min to 3 h (Durling et al., 2007; Jalbani et al., 2006; Rodrigues and Pinto, 2007). In light of these observations and knowing that the conditions for ultrasound-assisted extraction have both positive and negative interactions with each other, it is necessary to optimize conditions for each extraction. In this setting, an orthogonal design was the method of choice for this research.

Orthogonal array designs have been used very efficiently to discover how different parameters interact and how they affect product recovery (Stenlund et al., 2009). Previous knowledge of the variables, past experiences and intuition are all very helpful in arranging the variables and levels of the experiment because orthogonal array designs only cover a predefined region (Wang et al., 2008). This type of optimization procedure requires the use of a strategically designed experiment that deliberately introduces changes to identify factors affecting the procedure to estimate the factor levels required to yield an optimum response (Diez et al., 2008). Orthogonal here refers to balanced and separable and as such, when the effect of a factor is calculated, the influence of other factors is removed, with the result that different effects can be extracted independently. Up to 31 factors can be investigated; the precise number of factors is decided by the size of the trials, the complexity of the system and the level of detail which one requires of the information (Hedayat et al., 1999).

In this study, a new approach was developed that determined the overall best parameters for the UAE setting. Eight factors were included for global optimization, including three with four levels and five with two levels, so that the full factorial of $43 \times 25 = 2,048$ points was evaluated to find the best point (the optimum conditions). Using a mixed orthogonal array, the same optimal point was found using 0.78% of the available data set (16 out of 2,408 points) for these evaluations (Hedayat et al., 1999), as demonstrated in an industrial investigation (Kuo and Wu, 2009).

Although, previously reported ultrasound-assisted extractions employed orthogonal design, they focused mostly on models with three (Chen et al., 2010) or four (Chen et al., 2009) factors. Because of many controllable factors influencing extraction efficiency, there is no significant information gained from a larger number of factors (conditions). Therefore, this research employed an orthogonal design to evaluate eight factors that were critical to efficient extraction to determine their significance and thus, optimize the extraction process.

MATERIALS AND METHODS

Plant material preparation

Five-year-old Puna chicory roots, freshly harvested in September 2009 after growing in an experimental field of the Grassland Science Department, Northwest A&F University, Shaanxi Province, China, were washed several times with water, cut into pieces 3 to 5 mm in thickness and dried in an oven at 50°C until a constant weight was obtained (Chen et al., 2009). The dried material was then, ground into powder and passed through a 40 mesh sieve with a particle diameter of less than 0.35 mm (Diouf et al., 2009). Chicory root powder was then prepared and stored in sealed bags for future use. Ethanol (100%) and distilled water were supplied by the Experimental Materials Supplier Services Department of Northwest A&F University.

Apparatus and procedure

An electro-thermal constant temperature blast oven (DHG-9140A,

Table 1. Assignment of control factors and levels in the experimental design using a mixed orthogonal matrix (L_{16} ($4^3 \times 2^6$)).

Factor*	A (v:v, %)	B (v:w)	C (℃)	D(h)	E(repetitions)	F(min)	G(W)	H(repetitions)	I
Level I	0	8	20	24	1	30	200	1	
Level II	50	16	35	48	2	120	400	2	
Level III	75	24	50						
Level IV	100	32	65						

*Columns A, B, C, D, E, F, G, H and I stand for ethanol content, solvent-to-solid ratio, ultrasound temperature, impregnation time, impregnation repetitions, sonication time, ultrasound input power, sonication repetitions and vacancy to account for the statistical error, respectively.

Shanghai Yiheng Instrument Co., Ltd., China) with a temperature range from 10 to 250℃ was used in this study. A Chinese herbal medicine mill (FW177, Tai Si Te of Tianjin Instrument Co., Ltd. China) with a maximum speed of 24000 r/min and a particle range between 60 and 200 mesh, was also used. Furthermore, a NC ultrasound generator (KQ-500DE, Kunshan Ultrasound Instrument Co., Ltd., China) was also used in this study. The main technical data for the generator included a sonication bath capacity of 22.5 L (500 × 300 × 150 mm) with water inlet and outlet valves, a fixed frequency of 40 kHz, an adjustable sonication input power from 200 to 500 W, an adjustable temperature range from 10 to 80℃, a maximum automatic input heating power of 800 W and a maximum working time of 480 min. A multi-purpose vacuum pump (SHB-III, Zhengzhou Science and Industrial Foreign Trade Co., Ltd., China) recycle water during the experiment to maintain the water temperature between 4 and 10℃ with a vacuum limit of 0.098 MPa. Additionally, an electronic analytical balance and an electronic balance (YP1200, Shanghai Science and Industrial Co., Ltd., China) were also utilized in this study.

Experimental design

The experiment was designed to screen extraction conditions for global optimization and to determine the effects of operation conditions on the extraction of chicory root with ethanol. An L16 (43×26) mixed orthogonal array was adopted to assign eight factors and one vacancy (Hedayat et al., 1999).

Frequency is an important and complicated factor in UAE (Rodriguez et al., 2005) because multi-frequency extractions have been shown to be more effective than single frequency extractions. For example, sonication at 80 kHz facilitated the extraction of biochanin A and trans-resveratrol, while 25 kHz was effective in the extraction of daidzein and genistein from peanuts (Chukwumah et al., 2009). In addition, an established mathematical kinetic model for component extraction from *S. miltiorrhiza* proved that the extraction course was in line with ordinary extraction dynamics under dual-frequency ultrasound (Bi et al., 2010). Furthermore, several reports have applied ultrasonic energy at dual-frequencies or multi-frequencies for analytical chemistry research (Carreira et al., 2008; Fernandes et al., 2008). Therefore, frequency is not an investigational factor beyond this unique optimal condition study considering that high-power, low-frequency ultrasound in the range from 20 to 60 kHz has widespread clinical applications (O'Daly et al., 2008). This study utilized fixed, powerful ultrasonic working frequency at 40 kHz (Castagnede et al., 2008; Zhang and Liu, 2008).

Eight key factors that significantly influenced the extractive efficiency of UAE (aside from frequency) were investigated. The first three factors were placed into four levels; these included ethanol content (volume ratio: v/v; factor A) at 0, 50, 75 and 100%, solvent-to-solid ratio (v/w; factor B) at 8:1, 16:1, 24:1 and 32:1 and sonication temperature (factor C) at 20, 35, 50 and 65℃. The remaining factors were split into two levels, including impregnation time (factor D) with ethanol solvent at 24 and 48 h, impregnation repetitions (factor E) either once or twice, filtrate collection and extraction of the solid (when impregnation was performed twice) with the same volume of fresh solvent (Huang et al., 2009), sonication time (factor F) of either 30 or 120 min, an ultrasonic input power (factor G) of either 200 or 400 W and sonication repetitions (factor H) either once or twice. The assignment of the eight factors and their magnitudes are shown in Table 1, the spare column I was left vacant to account for the statistical error of the orthogonal method (Hedayat et al., 1999).

Preparation of crude extracts

The orthogonal matrix L_{16} ($4^3 \times 2^6$) led to 16 treatments under different conditions. Five hundred grams of chicory root powder was used in every experiment. The powder was put into jars, the corresponding ethanol solution was added and the mixture was stirred with a glass rod. Suspensions were then impregnated at room temperature for the remaining extraction time. A thermometer was put into the jar and the sample was sonicated at 40 kHz in the ultrasonic water bath with cooling water flowing from inlet to outlet valve. This cool water was adjusted to maintain the designated temperature of the extracting solution. When sonication was performed twice, the impregnation time was interrupted in the middle for the second sonication (that is, if the impregnating time was 24 h, it was sonicated at the 12 and 24^{th} h). After impregnation and sonication, the mixtures were filtered through two layers of filter paper and the filtrate was collected and rotary-evaporated in a vacuum at 40℃ (Jimoh et al., 2010). To evaluate the yield of the chicory, the extracts were weighed and the weights were converted using the following formula.

Extraction percentage (%) = (concrete chicory weight (g) /chicory root powder weight (g)) × 100%

Statistical analysis

Statistical analyses were conducted using the Statistical Analysis System (SAS) software (version 8.2, USA) (SAS-Institute-Inc, 1999). Trends were considered significant when the mean values of the compared sets were different at $P < 0.05$.

RESULTS

The results of visual analyses

The results of the L_{16} ($4^3 \times 2^6$) orthogonal experimental

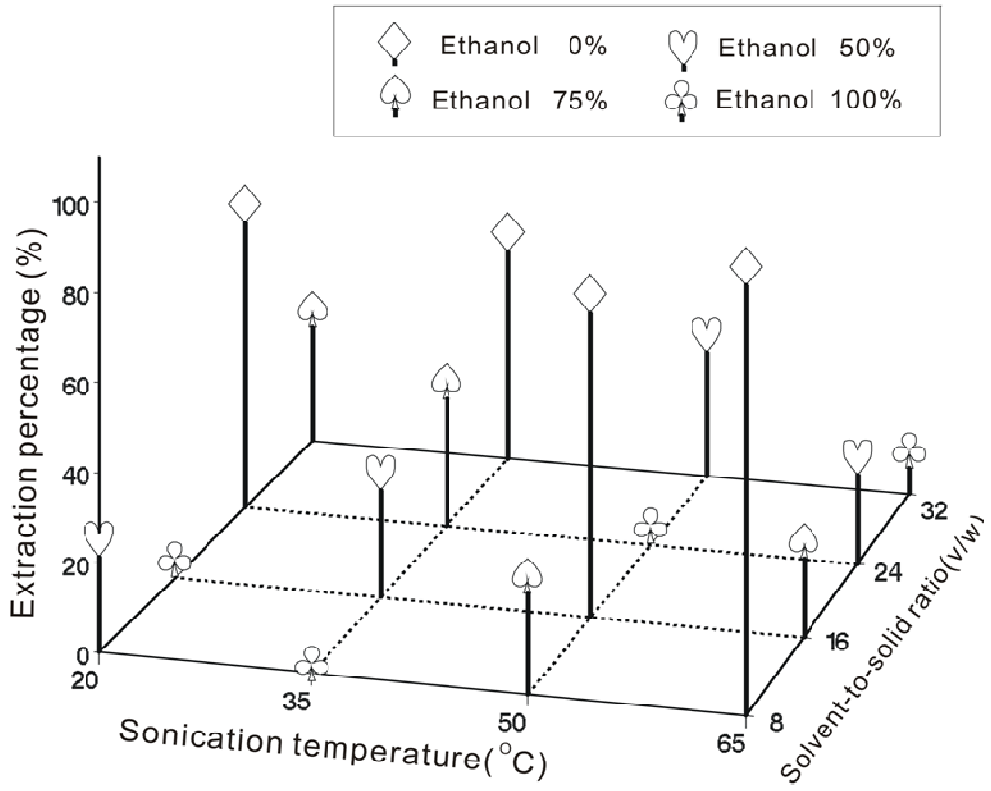

Figure 1. The effect of sonication temperature and solvent-to-solid ratio on extraction percentages.

Table 2. Assignment of factors and levels in the experiment using a $L_{16}(4^3 \times 2^6)$ matrix along with the response (extraction percentage).

Factor treatment	A(v:v, %)	B(v:w)	C(°C)	D(h)	E(repetitions)	F(min)	G(W)	H(repetitions)	I	Extraction percentage*(%)
1	1(0)	1(8)	4(65)	1(24)	2(2)	1(30)	2(400)	2(2)	1	**97.36**
2	2(50)	1	1(20)	2(48)	1(1)	1	1(200)	2	2	25.20
3	3(75)	1	3(50)	2	2	2(120)	1	1(1)	1	25.89
4	4(100)	1	2(35)	1	1	2	2	1	2	2.04
5	1	2(16)	3	2	1	2	2	2	2	72.96
6	2	2	2	1	2	2	1	2	1	28.32
7	3	2	4	1	1	1	1	1	2	21.50
8	4	2	1	2	2	1	2	1	1	4.29
9	1	3(24)	1	1	2	2	1	1	2	71.64
10	2	3	4	2	1	2	2	1	1	24.60
11	3	3	2	2	2	1	2	2	2	33.94
12	4	3	3	1	1	1	1	2	1	4.39
13	1	4(32)	2	2	1	1	1	1	1	54.60
14	2	4	3	1	2	1	2	1	2	33.80
15	3	4	1	1	1	2	2	2	1	32.09
16	4	4	4	2	2	2	1	2	2	10.43

* The highest extraction percentage is highlighted in bold.

design are shown in Figure 1 and Table 2. These results showed that treatments 1, 5, 9 and 13, which had the same 0% of ethanol (A_1, diamonds in Figure 1), showed extraction percentages in the decreasing order of 97.36,

Table 3. Averages of the levels together with the factors and ranges.

Factor *	A	B	C	D	E	F	G	H	I
Level I	**74.14**	**37.62**	33.31	**36.39**	29.67	**34.39**	30.25	29.80	33.94
Level II	27.98	31.77	29.73	31.49	**38.21**	33.50	**37.64**	**38.09**	33.94
Level III	28.36	33.64	34.26						
Level IV	5.29	32.73	**38.47**						
Range	68.85	5.86	8.75	4.90	8.54	0.89	7.39	8.29	0.00
Order**	1	6	2	7	3	8	5	4	9

*The highest extraction percentage of the levels is highlighted in bold; **It is the ordinal numeral for the range sequence of the eight factors in decreasing order.

Table 4. Sum of the levels together with the factors and the ranges.

Factor*	A	B	C	D	E	F	G	H	I
Level I	**296.56**	**150.49**	133.22	**291.14**	237.38	**275.08**	241.97	238.36	271.54
Level II	111.92	127.07	118.90	251.91	**305.67**	267.97	**301.08**	**304.69**	271.51
Level III	113.42	134.57	137.04						
Level IV	21.15	130.92	**153.89**						
Range	275.41	23.42	34.99	39.23	68.29	7.11	59.11	66.33	0.03
Order**	1	7	6	5	2	8	4	3	9

*The highest extraction percentage of the levels is highlighted in bold; ** It is the ordinal numeral for the range sequence of the eight factors in decreasing order.

72.96, 71.64 and 54.60%, which was much higher than that of the other treatments. Factor B, by comparison, increased from 8:1 to 32:1.

The extraction percentages of treatments 4, 8, 12 and 16, in increasing order were 2.04, 4.29, 4.39 and 10.43% at an ethanol content of 100% (A_4, clubs in Figure 1) as the solvent-to-solid ratio increased.

According to the orthogonal method (Bagheri et al., 2000; Chi and Bloebaum, 1996; Hedayat et al., 1999), the conditions that respond with the highest yield are the visual optimum conditions. The highest extraction percentage was observed in treatment 1 (97.36%, bolded in Table 2), which occurred at visually optimal conditions, as was the case for the treatment A1, B1, C4, D1, E2, F1, G2 and H2.

Range and variance analyses

The ranges of the average and sum of the factors are shown in Tables 3 and 4, respectively. The ranges in column I, which had statistical errors of 0 and 0.03 in Tables 3 and 4, respectively, indicated that the experiment was statistically accurate given its designed intention (Hedayat et al., 1999). Furthermore, according to the orthogonal method, as the range increases, the factor becomes more important (Bagheri et al., 2000; Chi and Bloebaum, 1996; Hedayat et al., 1999). As such, the ranges for the averages in decreasing order were A, C, E, H, G, B, D and F (indicated in the last row in Table 3) and the ranges for the sums in decreasing order were A, E, H, G, D, C, B and F (indicated in the last row in Table 4). This ordering of factors (Tables 3 and 4) thus, conveyed their relative importance. Additionally, the results showed that the eight factors (not including I) were significantly different according to variance analysis (PROC ANOVA) (Table 5).

Optimized conditions

According to the orthogonal method, the level of the averages (or sum) with the highest yield corresponded to optimum conditions (Hedayat et al., 1999). Both in terms of average and sum, the highest extraction percentage of the levels (optimal conditions) are highlighted in bold in Tables 3 and 4 (including level one of A and B and level four of C, for factors A, B and C). Accordingly, the optimal conditions for calculation were A1, B1, C4, D1, E2, F1, G2 and H2.

Both visually (treatment 1 in Table 2) and by calculation (Tables 3 and 4), the optimum conditions for the extraction were A1, B1, C4, D1, E2, F1, G2 and H2, which corresponded to an ethanol content of 0% (A1), a solvent-to-solid ratio of 8:1 (B1), a sonication temperature of 65°C (C4), an impregnation time of 24 h (D1) performed twice (E2), a sonication time of 30 min (F1), an ultrasonic input power (G2) of 400 W and sonication performed twice (H2).

Because the mixed orthogonal matrix is a uniform

Table 5. Variance analysis of the experimental factors.

Variation source	Sum of Squares	Degree of freedom	Mean Square	F value	$F_{0.01(m, n)}$
A	10014.88	3	3338.29	4768989.67 *	5403
B	79.33	3	26.44	37777.43 *	
C	155.26	3	51.75	73934.05 *	
D	96.19	1	96.19	137410 *	4052
E	291.47	1	291.47	416386*	
F	3.16	1	3.16	4513 *	
G	218.37	1	218.37	311963*	
H	274.98	1	274.98	392827*	
I (error)	0.0007	1	0.0007		
Total variation	11133.64	15			

*Statistically significant at 0.01.

Table 6. Average of the levels of the factors and the ranges without factors A or I.

Factor*	B	C	D	E	F	G	H
Level I	51.09	57.29	115.71	103.39	114.44	100.91	105.79
Level II	49.82	59.69	109.63	121.95	110.9	124.43	119.55
Level III	58.54	**62.26**					
Level IV	**65.89**	46.10					
Range	16.07	16.16	6.08	18.56	3.54	23.52	13.76
Order**	4	3	6	2	7	1	5

*The highest extraction percentage of the levels is highlighted in bold; **It is the ordinal numeral for the range sequence of the eight factors in decreasing order.

distribution (Bagheri et al., 2000; Chi and Bloebaum, 1996; Hedayat et al., 1999), the conditions A1 and A4, which indicated 0 and 100% ethanol content, were eliminated from Table 2 to investigate the optimal conditions for factors B through H again. Without A1 and A4, the ranges in decreasing order were G, E, C, B, H, D and F and the optimal conditions were B4, C3, D1, E2, F1, G2 and H2 (Table 6). In this case, the conditions D1, E2, F1, G2 and H2, were the same as the previous result; therefore, factors D through H were determined to be optimized (Hedayat et al., 1999).

Without A1 and A4, the average extraction percentage of level four of B and level three of C were the largest in their respective levels (highlighted in bold in Table 6). These conditions had the second highest extraction percentage (33.80%, underlined in Table 2) and corresponded to treatment 14. Therefore, the optimal conditions for calculation of factors B and C were B4 C3 (a solvent-to-solid ratio of 32:1 and a sonication temperature of 50°C).

DISCUSSION

The results of the experiments are reliable because the ranges of vacancy (column I) were close to zero (Hedayat et al., 1999). Based on this observation, it could be inferred that the mixed orthogonal matrix successfully screened the conditions for ultrasound-assisted extraction. Additionally, the ordered ranges implied the importance of the factors, as shown in Table 3, Table 4 and Table 6. This discovery was a new contribution to the existing research on UAE. Overall, the sum of the three ordered ranges suggested the importance of the factors in decreasing order to be A, E, G, C, H, B, D and F.

The optimum amount of ethanol varied widely in scientific reports. For example, it was calculated at 9% (v/v) for ursolic acid (Li et al., 2009), 70% (v/v) for extracting essential oils (Velickovic et al., 2008), 40% for extracting flavonoid (Huang et al., 2009), 98% for extracting betulin from *Broussonetia papyfera* bark (Chen et al., 2009), 61.09% for procyanidin (Wang et al., 2008) and 40% for tannins (Da Cunha et al., 2009). This wide range of optimized values possibly relates to the different aims of the different reports. Because a larger portion of ethanol makes the extracting solution less polar (Lu, 2004), essential oils, organic acids, resins and chlorophyll are all expected to be extracted at ethanol contents higher than 95%; however, when the ethanol content ranges from 50 to 70%, the extraction of alkaloids and glycosides is expected to be more effective. At ethanol contents less than 50%, bitter substances, anthraquinones and many drugs

Table 7. Sonication temperature and sonication time were reported in the literatures.

Sonication temperature (℃)	References	Sonication time (min)	References
25	(Ozcan et al., 2009)	5	(Ozcan et al., 2009)
28	(Ding et al., 2009)	5 to 20	(Jalbani et al., 2006)
30	(Dong et al., 2010)	12	(Yang et al., 2008)
31 to 34	(Ma et al., 2008)	15.1	(Guo et al., 2009)
40	(Durling et al., 2007; Khan et al., 2010)	20	(Li et al., 2007)
45 to 53	(Li et al., 2007)	25	(Dong et al., 2010)
45.64	(Wang et al., 2008)	29.1	(Zhang and Liu, 2008)
50	(Chen et al., 2009)	30	(Wang et al., 2007; Zhang et al., 2008; Zhao et al., 2007)
55	(Diao et al., 2009)	35	(Lin et al., 2009)
57	(Yang et al., 2008)	70	(Huang et al., 2009)
60	(Wu et al., 2009)	75	(Chen et al., 2010)
65	(Lin et al., 2009)	150	(Fu et al., 2006)
70	(Teng et al., 2009)		
80	(Zhao et al., 2007)		
81.5	(Wang et al., 2007)		
86.4	(Zhang and Liu, 2008)		
90	(Chen et al., 2010)		

can be suitably extracted (Lu, 2004). Taking this trend into consideration and knowing that chicory roots are cultivated for their inulin (Figueira et al., 2004), the ethanol content in this study had the most important role because the extraction percentages were much higher at 0% ethanol (A1) than at 100% (A4). As such, A1 and A4 acted as the extremes, while the optimized conditions were in the range from A2 to A3. Furthermore, factors D through H were demonstrated not to be related to ethanol content because the same optimum conditions of D1, E2, F1, G2 and H2 were obtained with or without factor A (ethanol content). Specifically, without A1 and A4, the optimal conditions for factors B and C were B4 and C3, respectively.

The relationship among factors A, B and C was complicated, as revealed in Figure 1. For example, at lower levels of A and B, C was higher, as indicated in treatment 1, whereas higher B required lower C (treatment 14). In addition, levels of factor partly depended upon the goal of the extraction. Ethanol content ranged from 55 to 75% (v:v) for a solvent-to-solid ratio of 6:1 and a sonication temperature of 40℃ for the extraction of three active compounds (Durling et al., 2007), whereas, at an ethanol content of 98%, a solvent-to-solid ratio of 42:1 and a sonication temperature of 50℃, betulin was extracted from *B. papyfera* bark (Chen et al., 2009).

The optimal solvent-to-solid ratio (factor B) of 32:1 found in this study was completely comparable to findings in the literature that included 20:1 (Dong et al., 2010), 34.4:1 (Huang et al., 2009), 40:1 (Fu et al., 2006), 60:1 (Yang and Zhang, 2008), 6:1 for three active compounds (Durling et al., 2007), 10:1 for tannins (Da Cunha et al., 2009) and 49:1 for polysaccharides (Lu et al., 2009). In this experiment, the solvent-to-solid ratio of 8:1 was likely the point of saturation for the distilled solvent (A1) or perhaps it indicated the influence of the ultrasound, because the effects of the ultrasound primarily involved superficial tissue disruption, accelerated surface mass transfer (Balachandran et al., 2006; Ji et al., 2006), increased yield of extracted components, decreased time of UAE (Fu et al., 2006), simplified manipulation, reduced solvent consumption, lower temperature and lower energy input (Khan et al., 2010). Furthermore, factor B at 8:1 occurred under condition A1 (0% ethanol or 100% distilled water) whereas, the optimum B values of 24:1 and 32:1 occurred with treatments 11 and 14, respectively (Table 2).

Sonication temperature (factor C), though variously reported in the literature as between 25 and 90℃ (list in Table 7), was calculated at 50℃ without factor A. As such, this factor could be inferred to be 50℃ for optimal extraction. Additionally, the optimal impregnation time (factor D) was determined to be 24 h, which was consistent with previous studies (Sandra et al., 2009; Wang et al., 2007). This observation suggested that the extraction solvent was likely saturated after a period of 24 h. Furthermore, the impregnation repetitions (factor E) were optimized at two in this study, as was performed in previous reports (Diouf et al., 2009; Li et al., 2009).

The results of this research indicated that, sonication time (factor F) was also an important factor and was optimized at 30 min, which was consistent with other reports

(Wang et al., 2007; Zhang et al., 2008; Zhao et al., 2007), whereas reports in the literatures ranged from 5 to 150 min (list in Table 7). One study put forth that, sonication repetitions (factor H) performed for three rounds of sonications was more efficient than one (Yang and Zhang, 2008). This report only partly confirmed the results found here, which was optimized to two rounds of sonications. In addition, ultrasonic input power (factor G) was optimized at 400 W in this study, which was also in line with the literature (Li et al., 2009). These observations, combined with the rest of the optimized conditions, implied that factors D through H were independent of A, B and C (Hedayat et al., 1999).

Conclusions

This study demonstrated that mixed orthogonal matrix design is an accurate, fast and economical tool for optimizing the conditions for ultrasound-assisted extraction. The importance of the eight factors in ultrasound-assisted extraction in decreasing order are ethanol content, impregnation repetitions, ultrasonic input power, sonication temperature, sonication repetitions, solvent-to-solid ratio, impregnation time and sonication time. At a frequency of 40 kHz, the optimal ultrasonic extraction conditions were an impregnation time of 24 h, with impregnation performed twice, a sonication time of 30 min and an ultrasonic input power of 400 W with sonication performed twice. These parameters were found to be independent of alcohol content, the solvent-to-solid ratio and the sonication temperature. At a frequency of 40 kHz, the alcohol content was optimized to range from 50 to 75% (v/v), the solvent-to-solid ratio was optimized to 32:1 and the sonication temperature was optimized to 50°C.

ACKNOWLEDGEMENTS

The Ministry of Science and Technology of the Peoples Republic of China, International Cooperation Research Project (2008DFA31650) and Shaanxi Province International Cooperation Research Project (2008KW-29) funded this work. We are grateful to Prof. Hu Tianming and postgraduate students Sun Luanzhi, Wu Chunhui and Du Jinhong. Also, we are grateful to Prof. S. Koda, Nagoya University, Nagoya, Japan, for critical review of this manuscript. Additionally, we greatly appreciate to the anonymous reviewers for their commenting on it.

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